JOURNAL OF BONE AND MINERAL RESEARCH
Volume 14, Number 3, 1999
Blackwell Science, Inc.

Thy-1 Antigen Expression by Cells in the Osteoblast Lineage

XIAO-DONG CHEN,[1] HE-YING QIAN,[1] LYNN NEFF,[1] KAZUHITO SATOMURA,[2] and MARK C. HOROWITZ[1]

ABSTRACT

Identification of surface markers involved in osteoblast differentiation provides a method to isolate osteoblasts at various stages of maturation. In this study, we examined expression of the T lymphocyte differentiation antigen, Thy-1, by osteoblastic cells from different species. Murine skeletal progenitor, neonatal calvarial, and adult bone cells (ABCs) were selected to represent osteoblasts at distinct stages of maturation. Flow cytometric analysis showed that Thy-1 expression was undetectable on the progenitor cells (mouse limb bud clones 14 and 17), appeared on calvarial cells (45%$^+$), and was decreased on ABCs (< 10%$^+$). Thy-1 was also detected in situ on osteoblastic cells in mouse calvariae. Thy-1 mRNA expression correlated with cell surface expression. Antigen expression was markedly increased during the cells' proliferative phase in culture. Furthermore, examination of primary rat and human osteoblast-like cells revealed that significant levels of Thy-1 were also expressed on those cells derived from subconfluent culture. This study indicates that osteoblasts express Thy-1 antigen and that its expression is maximal at their earliest stage of maturation, during the proliferative phase, and then declines as the cells mature. In a role similar to the one it plays in the hematopoietic system, Thy-1 antigen may be useful as a differentiation marker in following the development of the osteoblast. (J Bone Miner Res 1999;14:362–375)

INTRODUCTION

It is believed that osteoblasts arise from mesenchymal stem cells and that progeny of these stem cells pass through a series of maturational stages on their way to becoming mature functional cells in a manner similar to hematopoietic cell differentiation.[1–4] However, little is known about this pathway of maturation, particularly when compared with hematopoietic cell differentiation. It has been difficult to follow osteoblast differentiation because of the inability to isolate and characterize cells at discrete stages along the lineage. One way to trace osteoblast differentiation is to identify cell surface protein antigens which are specific for these stages of differentiation. Identification of such surface determinants provides a method to isolate cells and more effectively study their biological activities as a function of development.

There is some information on the antigenic phenotype of osteoblasts (reviewed in Ref. 5).[5] A number of attempts by different investigators have been made to raise antibodies to cell surface protein antigens of osteoblast-like cells and in many cases antibodies to alkaline phosphatase (ALP) have been generated.[6–9] Bruland et al. used a human osteosarcoma cell line to induce a monoclonal antibody (MAb) which recognized an 80 kDa antigen expressed on normal osteoblasts, which was absent on osteocytes.[6] In chickens, antibodies specific for osteocytes have also been described.[7,10] These antibodies appear to be different from a series of antibodies that distinguishes preosteoblasts from more mature cells.[7] However, the antigens recognized by these antibodies are unknown. It was also found that CD44, a transmembrane glycoprotein which can serve as an adhesion and homing receptor with specificity for hyaluronate, was expressed on human osteocytes, osteoclasts, and periosteal cells, but not on osteoblasts or lining cells.[11] The HOB-1 antibody labels hypertrophic chondrocytes, osteoblasts, and osteocytes.[12] An osteocyte-specific MAb raised against an unknown epitope has been used to

[1]Department of Orthopaedics and Rehabilitation, Yale University School of Medicine, New Haven, Connecticut, U.S.A.
[2]The National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, U.S.A.

positively select and study these cells in chickens.[13] We have reported that murine osteoblasts express two of the Ly-6 differentiation antigens (Ly-6 A and Ly-6C), which can be regulated by osteotropic agents including interferon, transforming growth factor β-1, and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$).[14] Ly-6 alloantigens are glycosylphosphatidylinositol (GPI) anchored membrane glycoproteins with molecular weights of between 15 kDa and 18 kDa.[15,16] The Ly-6 multigene family encodes differentiation antigens originally detected on lymphoid cells and on hematopoietic stem cells.[17,18] Sca-1 (stem cell antigen), an antigen found on murine hematopoietic stem cells, is now known to be Ly-6 A.2.[18] Sca-1 is also found on murine bone marrow derived osteoprogenitor cells.[3]

MAbs SB10 and SB20, raised against unknown determinants on human mesenchymal stem cells, apparently recognize at least two populations of bone cells in the osteogenic lineage.[19] The ALP^- $SB\text{-}10^+$ population was found in the outer layer of the periosteum in fetal calvariae and long bones, sites consistent with osteoprogenitor cells. SB-10 immunoprecipitates the cell surface glycoprotein antigen activated leukocyte-cell adhesion molecule from human osteoprogenitors.[20] Activated leukocyte-cell adhesion molecule is a member of the immunoglobulin (Ig) super family of cell adhesion molecules with a MW of ~99 kDa. The ALP^+ $SB\text{-}20^+$ population was composed of the osteoblastic cells in the inner periosteal layer, a site consistent with more mature osteoblastic cells. These reports suggest that in the osteoblast lineage, there exist specific surface antigens which may be used for studying osteoblast differentiation.

Thy-1 is a member of the Ig supergene family with structural similarity to the Ig V_H region domain.[21] The molecule is a 25–30 kDa GPI-linked membrane glycoprotein encoded by a single gene on mouse chromosome 9.[22,23] In mouse, Thy-1 exists in two allelic forms, Thy-1.1 and Thy-1.2, which differ by a single amino acid substitution of glutamine for arginine at residue 89.[24] In humans and rats, Thy-1 is nonallelic. It is expressed in a tissue-specific and developmentally controlled manner. Thy-1 is expressed on thymocytes, peripheral T cells, fibroblasts, epithelial cells, neurons, and hematopoietic stem cells.[23–25] In mouse, the initially high levels of expression of the protein on thymocytes and peripheral T cells decrease with differentiation. Only a small population of bone marrow cells (2–7%) express Thy-1. The expression of Thy-1 has been used to help identify and isolate hematopoietic stem cells in combination with other markers. For example, $Thy\text{-}1^{low}$ Lin^- $Sca\text{-}1^+$ bone marrow cells have been identified as hematopoietic stem cells giving rise to all blood cell lineages.[26,27] It has also been shown that Thy-1 is an activation determinant. Using MAbs to bind and cross-link Thy-1 on the T cell surface may result in T cell activation in a manner similar to that seen with plant lectins, leading to a polyclonal induction of interleukin-2 (IL-2) production, IL-2R expression, and proliferation.[28]

We have previously shown that treatment of the nontransformed murine osteoblast-like cell line MC3T3-E1 with anti–Thy-1 antibodies plus complement resulted in cell lysis, indicating expression of the antigen.[14] Therefore, we have examined in detail the expression of Thy-1 by cells in the osteoblastic lineage. In the present study, we found that mouse, rat, and human cells in the osteoblast lineage express Thy-1 antigen and that expression changes with differentiation. In addition, Thy-1 expression and regulation differs from that of other cell surface antigens. We propose that, as in the hematopoietic system, Thy-1 antigen expression on osteoblast-like cells may be used to help follow the development of the osteoblast lineage.

MATERIALS AND METHODS

Cells

Skeletal progenitor cell lines: Murine limb bud clones 14 and 17 (referred to as MLBs) are undifferentiated murine skeletal progenitor cells immortalized by v-*myc* transfection. Upon exposure to bone morphogenetic protein-2 (BMP-2), clone 14 differentiates into a chondroblast-like cell line, while clone 17 differentiates into an osteoblast-like cell line.[29] They grow continuously in the absence of added growth factors as adherent monolayers and are maintained by biweekly passage in 75-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum (NBCS). These cells were provided by Dr. Vicki Rosen (Genetics Institute Inc., Cambridge, MA, U.S.A.).

Murine bone marrow stromal cell lines: W20–17 (referred to as W20) is an osteoblast progenitor cell line isolated from the bone marrow of the W^{++} mouse strain. The osteoblast phenotype of W20 cells can be induced by treatment with BMP-2.[30] W20 cells were maintained by weekly passage in 75-cm^2 tissue culture flasks in DMEM with 10% NBCS and were also obtained from Dr. Vicki Rosen. BMS2 is a stromal cell line isolated from bone marrow by treatment of cultures with 5-fluorouracil. These cells secrete IL-6 and support B cell lymphopoiesis in vitro. BMS2 cells spontaneously undergo adipogenesis. However, these cells express genes characteristic of osteoblastic cells and respond to osteoinductive factors.[31–33] Thus, the BMS2 cell line provides an in vitro model of a multipotent stromal cell progenitor. The cells were maintained by weekly passage in 75-cm^2 tissue culture flasks in DMEM containing 1 mM sodium pyruvate and 10% NBCS. These cells were obtained from Dr. Jeff Gimble (Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.).

Marrow stromal fibroblasts: The femora and tibiae of 6- to 8-week-old C57BL/6 mice were dissected out, cleaned of adhering tissues, and washed three times with Hank's balanced salt solution (Life Technologies, Inc., Grand Island, NY, U.S.A.) containing 400 IU/ml penicillin and 400 mg/ml streptomycin (Biofluid, Inc., Gaithersburg, MD, U.S.A.). The epiphyses were removed, and the bone marrow was flushed from the bone shafts using alpha-modified essential medium (α-MEM) (Life Technologies, Inc.). A single cell suspension was obtained and filtered through a strainer (Becton Dickson Labware, Franklin Lakes, NJ, U.S.A.) to remove any tissue debris. The bone marrow cells were seeded into 75-cm^2 flasks (Falcon Labware, Meylan Cedex, France) at a cell density of 3×10^7 cells/flask, and cultured

in α-MEM containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, U.S.A.), 10^{-8} M dexamethasone (Sigma Chemical Company, St. Louis, MO, U.S.A.), 10^{-4} M L-ascorbic acid (phosphate magnesium salt; Wako Pure Chemical Industries, Ltd, Osaka, Japan), 100 IU/ml penicillin, and 100 mg/ml streptomycin. The culture medium was changed after the first week and then twice weekly thereafter. At 2 weeks of culture, the bone marrow cells were subcultured into 150-mm tissue culture dishes (Falcon Labware) at a cell density of $2–10 \times 10^4$ cells/dish. Only half of the culture medium was changed twice a week until the distinct colonies were identified. Thereafter, the medium was completely changed twice a week. Two to 3 weeks later, the discrete colonies were cloned using cloning cylinders (Sigma Chemical Company). Five out of 30 individual clones were used in the following investigations. These precursors may give rise to at least four types of connective tissue, including bone.[34]

Neonatal murine calvarial cells were prepared as previously described.[14] Briefly, calvariae from 3- to 5-day-old C57BL/6 mice were pretreated with 4 mM EDTA washes in phosphate-buffered saline (PBS) (3×10 minutes). The calvariae were subjected to sequential enzymatic digestion using CLS-2 bacterial collagenase (Worthington Biomedical Corp. Freehold, NJ) at 200 U/ml in PBS. Cells released after the first 10-minute digestion were designated fraction 1; cells released after the second 10-minute digestion were designated fraction 2; this procedure was continued through five sequential treatment periods. In the experiments presented here, fractions 1 and 2 were discarded and fractions 3–5 were pooled and served as the starting population of cells. The released cells in fractions 3–5 are highly enriched in proliferating cells consistent with osteoblast precursors and osteoblasts.[35] The cells were washed twice in culture medium (α-MEM with 10% NBCS), counted, and plated at low density in 100-mm dishes (1000–2000 cells/cm^2) and grown to confluence (5–7 days). Cells at confluence stop proliferating, display a low rate of DNA synthesis, and start to secrete matrix proteins,[36] but have not yet entered the mineralization phase.[37]

To directly follow Thy-1 expression by differentiating osteoblasts, an in vitro culture system was used.[37] Calvarial cells were seeded as described above and once confluence was reached (approximately day 7), the cultures were fed twice weekly with medium containing 2 mM β-glycerophosphate. The cells were collected from cultures on days 3, 5, 7, 14, and 28 after initial seeding and tested for Thy-1 expression by flow cytometry. Based on the changes of osteoblast phenotypic markers, the cells may be divided into three distinct stages: proliferation (days 3 and 5), matrix production (day 7 and 14), and mineralization (day 28).[37] The cells progress from a less mature to a more mature phenotype.

To determine whether Thy-1 antigen expression was cell cycle dependent, calvarial cells were treated with hydroxyurea. This agent inhibits DNA synthesis (> 95%) and therefore arrests cell cycle progression at the $G_1 \rightarrow S$ transition.[35] The calvarial cells were seeded into 100-mm dishes (1000–2000 cells/cm^2) and cultured until day 3 (proliferating cells) or day 7 (cells at confluence) and treated with 1 mM hydroxyurea (Sigma Chemical Company) for 24 h. Antigen expression was analyzed by flow cytometry.

Bone cells were isolated from adult murine long bones using a modification of the technique of Robey and Termine.[38] The tibiae and femora were obtained from 6- to 8-week-old C57BL/6 mice and cleared of soft and connective tissue. The periosteum was removed by scraping with a scalpel. The epiphyses were cut below the metaphysis, leaving the diaphysial shaft. After removing all of the visible bone marrow cells by flushing the marrow channel with PBS, the tibia and femora were placed into 2-ml conical vials and minced into fine chips. The chips were incubated twice with 250 U/ml of CLS-2 bacterial collagenase in DMEM for 40 minutes at 37°C. This procedure rendered all chip surfaces (periosteal, endosteal, and oblique) devoid of adherent cells as determined by scanning electron microscopy (M. Horowitz, unpublished data). The chips were washed and transferred to 100-mm tissue culture plates in α-MEM with 10% NBCS. Cells were allowed to grow out from the chips and form a subconfluent monolayer (80–90%). These cells have characteristics of mature osteoblast-like cells. They proliferate slowly as compared with calvarial osteoblasts, express ALP and osteocalcin, and do not respond to BMP. Although these cells are clearly in the osteoblast lineage and appear to be somewhere between calvarial osteoblast-like cells and true osteocytes in maturity, the precise phenotype and stage of differentiation is not defined. Therefore, they will be referred to as adult bone cells (ABCs).

Primary fetal rat calvarial osteoblasts (raOBs) were obtained from 22-day rat fetuses (Sprague-Dawley; Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) by five 20-minute sequential collagenase digestions, similar to the procedure described above. Populations 3–5 were pooled and seeded at low density (~1000 cells/cm^2) in DMEM supplemented with nonessential amino acids, penicillin, streptomycin (GIBCO BRL, Grand Island, NY, U.S.A.), 20 mM HEPES, pH 7.0, 10% FBS (Sigma Chemical Company), and 100 μg/ml L-ascorbic acid (Fisher Scientific, Pittsburgh, PA, U.S.A.), and incubated for 6–8 days, by which time the cells reached confluence. This population of cells is highly enriched with osteoblastic cells and osteoblast precursors.[39] These raOBs were provided by Dr. Michael Centrella (Yale University School of Medicine, New Haven, CT, U.S.A.).

Normal human bone cells were obtained using techniques previously described.[38] Normal human bone was obtained from patients (≥ 60 years old) who were undergoing surgery following accidental injury or hip replacement. Bone was cleared of soft tissue, and the trabecular bone was processed into small chips by mechanical reaming. The chips were washed in medium (DMEM without serum) and further minced with a microdissecting scissors and then a Polytron homogenizer. The minced bone chips were washed extensively with medium to remove blood components and then incubated in DMEM medium containing 250 U/ml of CLS-2 bacterial collagenase twice for 40 minutes in a shaking 37°C water bath to ensure removal of any attached cells. No adherent cells can be observed on chip surfaces as determined by scanning electron micros-

copy. The chips were washed and placed in 100-mm tissue culture dishes in 10 ml of α-MEM with 10% NBCS and cultured at 37°C. Cells grow from the chips and approach confluence in 3–4 weeks. They are then lifted from the dishes and separated from the chips using either collagenase or trypsin treatment. These cells express the osteoblastic or preosteoblastic phenotype.[40]

Monoclonal antibodies

Hybridoma G7.4.2 (rat anti–mouse Thy-1 antigen, IgG2c) was obtained from Dr. Jeffrey A. Bluestone (Ben May Institute, University of Chicago, Chicago, IL, U.S.A.).[41] JlJ is a rat anti-mouse Thy-1 MAb (IgM) obtained from Dr. Donal Murphy (New York State Department of Health, Albany, NY, U.S.A.).[42] Conditioned medium from antibody producing hybridomas was tested for specificity by flow cytometry using mouse thymocytes (100% positive), spleen T cells (65% positive), and bone marrow cells (< 10% positive). A rat anti-mouse Thy-1 MAb (IgG2a) directly conjugated to fluorescein isothiocyanate (FITC) was purchased from Pharmingen (San Diego, CA, U.S.A.) and used at 2 μg/ml. Mouse anti-rat Thy-1 antibody and mouse anti-human Thy-1 antibody were also purchased from Pharmingen. The concentrations of antibody used were: 1/10 dilution of G7.4.2 conditioned medium, 1/50 dilution of JlJ, 5 μg/ml of mouse anti-rat Thy-1 antibody, and 2.5 μg/ml of mouse anti-human Thy-1 antibody. The optimal concentrations were determined by dilutional analysis in pilot experiments.

Flow cytometry

Single-cell suspensions were prepared from monolayer cultures by trypsin treatment (0.02% for 2 minutes at 37°C) followed by two washes in cold PBS with 2% NBCS. For antibody staining, the cells ($5–10 \times 10^5$) were incubated in 100 μl of diluted anti–Thy-1 antibodies for 30 minutes at 4°C. The stained cells were washed twice in staining buffer (PBS with 5% NBCS and 0.01% sodium azide) and incubated in 20 μg/ml of FITC-labeled goat anti-mouse IgG for 20 minutes at 4°C, then washed twice with staining buffer and either immediately analyzed by flow cytometry or fixed with 1% paraformaldehyde in PBS and analyzed within 96 h. Cells were incubated with secondary antibody only as a negative control. The cell suspensions were analyzed using a Becton Dickinson FACStar^{Plus} flow cytometer. Excitation was at 488 nm and emission was collected at 525 nm. For each sample, 10,000 events were collected.[14] The percentage of positively stained cells was derived directly from the fluorescence-activated cell sorting (FACS).

Phosphatidylinositol-specific phospholipase-C

Neonatal murine calvarial cells were harvested from subconfluent cultures by trypsin treatment. Fresh thymuses from C57BL/6 mice were processed into single cell suspensions. Cells were washed in RPMI-1640 with 10 mM HEPES and then resuspended in this same medium ($1–5 \times 10^6$ in 1 ml) containing phosphatidylinositol-specific phospholipase-C (PI-PLC) (Sigma Chemical Co.) at 1 U/ml and incubated for 90 minutes at 37°C.[43] The cells were washed and then processed for flow cytometry. PI-PLC treatment of osteoblasts caused a 10-fold reduction in the expression of Ly-6, another GPI-linked antigen.[14]

Immunohistochemistry

As previously described,[44] calvariae from 2-day-old C57BL/6 mice were pretreated with 4 mM EDTA in PBS (3×10 minutes), then incubated with CLS-2 bacterial collagenase at 200 U/ml in PBS for 15 minutes twice at 37°C. Calvariae from four 2-day-old mice were fixed for 1 h in 3.7% formaldehyde at 4°C. Tissue was washed in PBS and infiltrated with 40% sucrose in PBS for 48 h at 4°C. Cryostat sections (6 μm) were prepared using a Bright Cryostat fitted with a Reichert Autocut microtome (Vienna, Austria). Sections were blocked in PBS with 0.1% BSA, 0.05% saponin, and 5% normal goat serum for 30 minutes. The sections were then incubated with primary antibody, a 1:10 dilution of G7.4.2, for 2 h at room temperature, then washed and incubated with FITC-conjugated secondary antibody for 1 h at room temperature. The sections were washed, mounted in Fluorsave (Calbiochem-Novabiochem Corporation, La Jolla, CA, U.S.A.), and examined using a confocal microscope (MRC 600; Bio-Rad, Melville, NY, U.S.A.) with a krypton/argon laser.

Measurement of ALP activity

Calvarial cells were plated in 100-mm dishes at 1000–2000 cells/cm^2. ALP activity was measured from cultures 3, 5, 7, 14, 28, and 35 days after initial seeding, which correspond to the time course for examining Thy-1 expression. Single cells were prepared from monolayer cultures by trypsin treatment and lysed in cell suspension at 10^6 cells/0.5 ml lysis buffer (20 mM Tris, 0.5 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 0.1% Triton X). The ALP levels in lysed cells were determined using an ALP kit (Sigma Chemical Co.), and production of ρ-nitrophenol was measured by spectrophotometric absorbance at 405 nm. The ALP value was calculated from four cultures using standards and expressed by Sigma units per 10^6 cells. One Sigma unit is equal to 1 μM ρ-nitrophenol/h. Data are presented as the mean ± SD.

Northern blot analysis

The probes were purified from their respective plasmid vectors by appropriate restriction endonuclease digestion, separated by agarose gel electrophoresis, and recovered from the agarose using Geneclean kit (BIO 101 Inc., LaJolla, CA, U.S.A.). A fragment of Thy-1 cDNA was released as an *Eco*RI insert carried in pUC19. Specific activities of greater than 2×10^9 cpm/μg were attained by random priming using both ^{32}P-dATP and ^{32}P-dCTP and a random primers DNA labeling kit (GIBCO BRL).

MLBs, calvarial osteoblasts, and ABCs were seeded at 1000 cells/cm^2 in 100-mm tissue culture dishes and grown to

~80% confluent density. The RNA was isolated using TRIzol Reagent (GIBCO BRL), a monophasic solution of phenol and guanidine isothiocyanate, according to the manufacturer's protocol. The RNA was quantitated by measuring absorption at 260 nm, and the concentration adjusted to 2 μg/μl with RNAse free water.

RNA (up to 20 μg) was denatured in glyoxol/dimethyl sulfoxide, electrophoresed on 1.5% agarose gels in 10 mM phosphate buffer, and transferred to Zetabind nylon membrane (Cuno, Meriden, CT, U.S.A.). Following transfer, the membranes were air dried for 30 minutes and cross-linked by exposure to short wave UV light for 3 minutes. Filters were prehybridized for 30 minutes at 65°C in hybridization buffer (0.5 M $NaPO_4$, pH 6.8, 7% SDS, 1% BSA, 1 mM EDTA) and then hybridized for 20 h with 5×10^6 cpm of labeled probe per milliliter of hybridization buffer.[45] After hybridization, filters were washed twice in buffer A (40 mM $NaPO_4$, 5% SDS, 0.5% BSA, 1 mM EDTA) for 15 minutes at 65°C and then washed two to four times in buffer B (40 mM $NaPO_4$, 1% SDS, 1 mM EDTA) for 15 minutes at 65°C. Then the filters were exposed to Kodak XAR film overnight at –70°C.

For rehybridization with another radiolabeled probe, the filters were placed in a 95°C solution of 0.1% SDS and 0.1× SSC (strip solution) and shaken for 20 minutes. This procedure was repeated three times using fresh strip solution. The filters were dried and exposed to film to confirm the lack of residual radiolabel.

Spleen T cell preparation

Spleen cells were separated into Ig^+ and Ig^- populations by panning on anti–Ig-coated plates as previously described.[46] Spleens from C57BL/6 (H-2^b) mice were removed under sterile conditions and processed into single cell suspensions. The red blood cells were lyzed by hypotonic shock, and the remaining cells were adjusted to 10^7 cells/ml in PBS with 2% FBS. Spleen cells (5×10^7) were incubated for 1 h at 4°C on plastic Petri dishes which had been coated with goat anti-mouse Ig. The nonadherent T cell enriched population was greater than 95% Thy-1^+ as determined by FACS analysis.[46]

RESULTS

Thy-1 antigen expression by the osteoblast lineage in the mouse

As representatives of stages of osteoblast differentiation from least to most mature, skeletal progenitor cells MLB clones 14 and 17, neonatal murine calvarial osteoblasts, and bone cells from adult long bones were examined for Thy-1 expression. In addition, Thy-1 expression on marrow stromal cell lines W20 and BMS2, and five marrow stromal fibroblast cell lines was examined. Antigen expression was analyzed by flow cytometry using monoclonal anti–Thy-1 antibody (G7.4.2). The histograms depicting the Thy-1 staining pattern of MLB clones 14 and 17 were indistinguishable from the negative controls (Figs. 1A and 1B), indicating that neither clone expressed detectable Thy-1 antigen. The marrow stromal cell line W20 and all five marrow stromal fibroblast cell lines also failed to express detectable Thy-1 (Figs. 1C and 1D). In contrast, once cells started to express the osteoblast phenotype, Thy-1 appeared. The majority of the BMS2 stromal cells (90%) expressed low levels of Thy-1 as indicated by the single peak being shifted to the right (Fig. 1E). Freshly isolated primary calvarial osteoblastic cells analyzed immediately after isolation, without additional culture, were 45% positive (range 35–50%) for Thy-1. However, the amount of antigen per cell was much more variable than that seen on BMS2 cells with many more brightly stained cells (Fig. 1F). Thy-1 antigen expression decreased dramatically on the bone cells isolated from adult long bones (passage 0). The Thy-1^+ population was < 10% of the total population with few brightly staining cells (Fig. 1G). These data suggest that Thy-1 expression was initially undetectable, appeared and then decreased with osteoblast development.

Immunohistological staining for Thy-1 antigen in neonatal murine calvaria

Although the Thy-1 antigen was expressed on freshly isolated osteoblastic cells, it was unclear whether Thy-1 was also expressed in situ. The distribution of Thy-1^+ cells in the calvaria was determined by immunohistological staining using confocal microscopy. Figure 2 shows a frozen sagittal section through the periphery of parietal calvaria. Osteoblasts are strongly positive for Thy-1 and located on the endocranial surface while lining cells, which also stain with Thy-1, are seen at the outer periosteal surface separating mineralized bone from cartilage. Cells in the cartilage are negative. Staining with secondary antibody alone, as a negative control, showed no staining (data not shown).

Thy-1 expression by differentiating murine calvarial osteoblasts

In addition to selecting cells at different stages of differentiation, we have used the well described in vitro culture system of calvarial osteoblastic cells to determine whether Thy-1 expression was related to osteoblast differentiation. We tested the cells during their proliferative stage (day 3), at confluence (day 7) when matrix proteins were starting to be secreted, and at the later phase of matrix maturation and mineralization (day 14 and 28). Using the same cells, we have measured ALP production to directly compare antigen expression with differentiation. Examination of the highly proliferative day 3 or day 5 cells revealed that 75% were Thy-1^+ (Fig. 3A). Once the cells had attained confluence and stopped proliferating (day 7), the number of positive cells decreased to 50% with an associated loss of brightly staining cells (shift to the left), indicating the amount of Thy-1 antigen expressed on the surface of cells was reduced significantly (Fig. 3B). As the cells progressed through the matrix maturation and mineralization stages, the number of Thy-1^+ cells continued to decrease to < 20% (Figs. 3C and 3D). As can be seen in Fig.

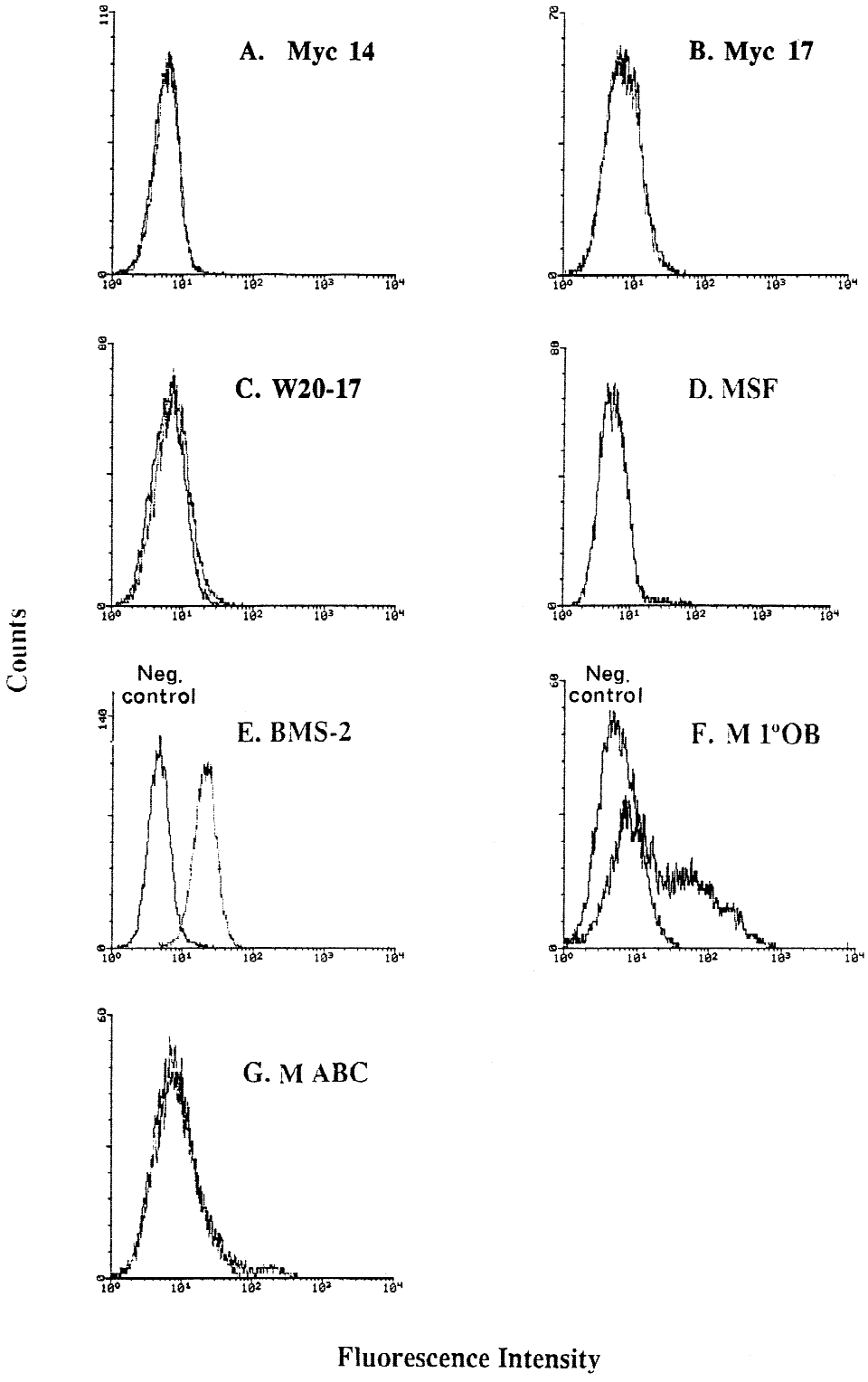

FIG. 1. FACS analysis of Thy-1 antigen expression by murine osteoblast lineage cells. Cells were grown to subconfluence, recovered by trypsin treatment, and stained with primary antibodies to Thy-1 antigen and a FITC-conjugated secondary antibody. (A) MLB 14, murine limb bud clone 14; (B) MLB 17, murine limb bud clone 17; (C) W20–17, murine marrow stromal cell line; (D) MSF, marrow stromal fibroblasts; (E) BMS2, murine marrow stromal cell line; (F) M 1°OB: primary neonatal murine osteoblastic cells (calvarial cells); (G) M ABCs, murine ABCs. Negative controls were included in all experiments. In (A–D), the histograms of the negative control and the Thy-1–stained cells were superimposable.

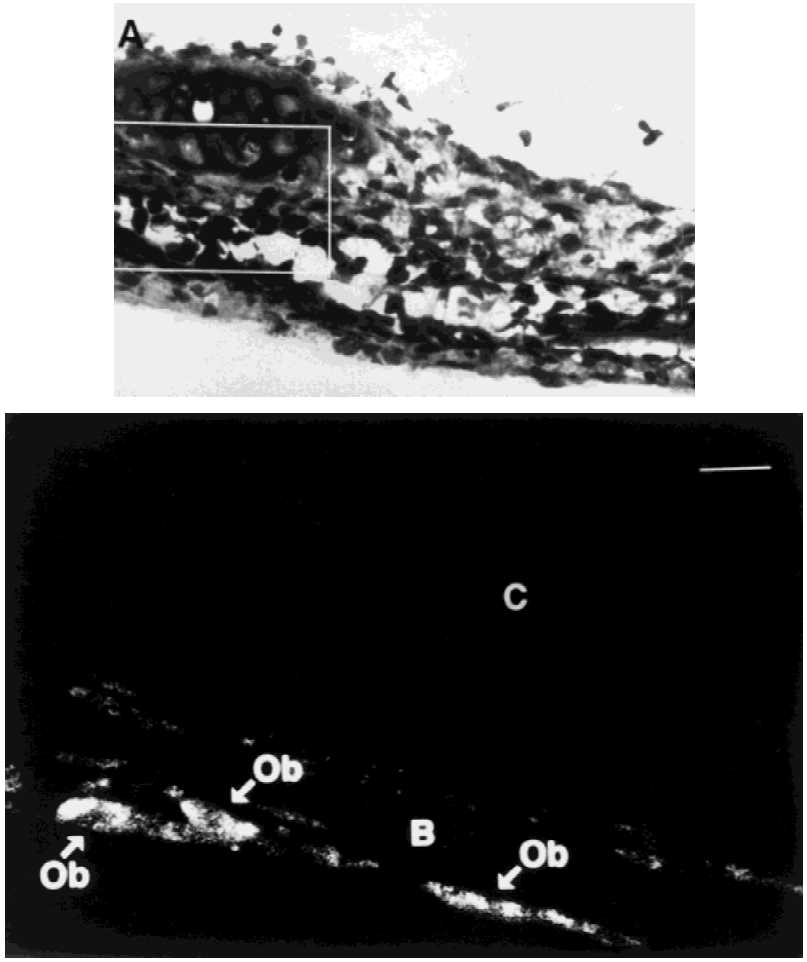

FIG. 2. Immunohistological staining for Thy-1 antigen in neonatal murine calvaria. The sections of calvariae from 2- to 3-day-old C57BL/6 mice were incubated with primary antibody, a 1:10 dilution of G7.4.2, for 2 h, then washed and incubated with FITC-conjugated secondary antibody for 1 h at room temperature. Incubation with FITC-conjugated secondary antibody for 1 h at room temperature alone served as a negative control (data not shown). (A) The histologic structure of a sagittal calvarial section stained with methylene blue. The box indicates the localization of immunolabeling on a serial section shown at higher power in the immunofluoruescent view. Osteoblasts (Ob) are strongly positive for Thy-1 and located on the endocranial surface while lining cells, which also stain with Thy-1 are seen at the outer periosteal surface separating mineralized bone (B) from cartilage (C). The bar represents 10 μm.

3E, ALP activity was low during the proliferative phase, increased after the cells reached confluence and peaked during matrix maturation and mineralization in a manner similar to fetal rat calvarial osteoblasts. These cells go on to form Von Kossa positive bone nodules measured at day 35 (data not shown). These data suggest that proliferative osteoblastic cells expressed higher levels of Thy-1 antigen than cells which had stopped dividing and had entered the phases of matrix maturation and mineralization. Moreover, it would appear that as the osteoblasts mature, as indicated by increased ALP expression, Thy-1 expression decreases.

An alternative explanation was that Thy-1 expression was really cell cycle dependent and only correlated with maturation. To determine whether the expression of Thy-1 antigen was cell cycle dependent, day 3 or day 7 calvarial cell cultures were treated with hydroxyurea to block DNA synthesis. Untreated day 3 cells were 75% Thy-1^+ as compared with 71% for hydroxyurea-treated cells (data not shown). Similar results were obtained for the day 7 cultured cells (data not shown). These data suggest that the expression of Thy-1 antigen was not cell cycle dependent.

To determine whether maintenance of the proliferative stage correlated with high Thy-1 expression, freshly isolated calvarial osteoblastic cells were compared with those that had been grown to subconfluence (day 5 of culture). As shown previously, freshly isolated primary calvarial cells were 45% Thy-1^+ (Fig. 4B, uncultured). However, the subconfluent cultured cells were greater than 75% Thy-1^+ with many more brightly staining cells (Fig. 4B, cultured). These same cells were grown to confluence and reseeded two additional times, and while the cells were subconfluent, their Thy-1 expression was always 75–85% positive (data not

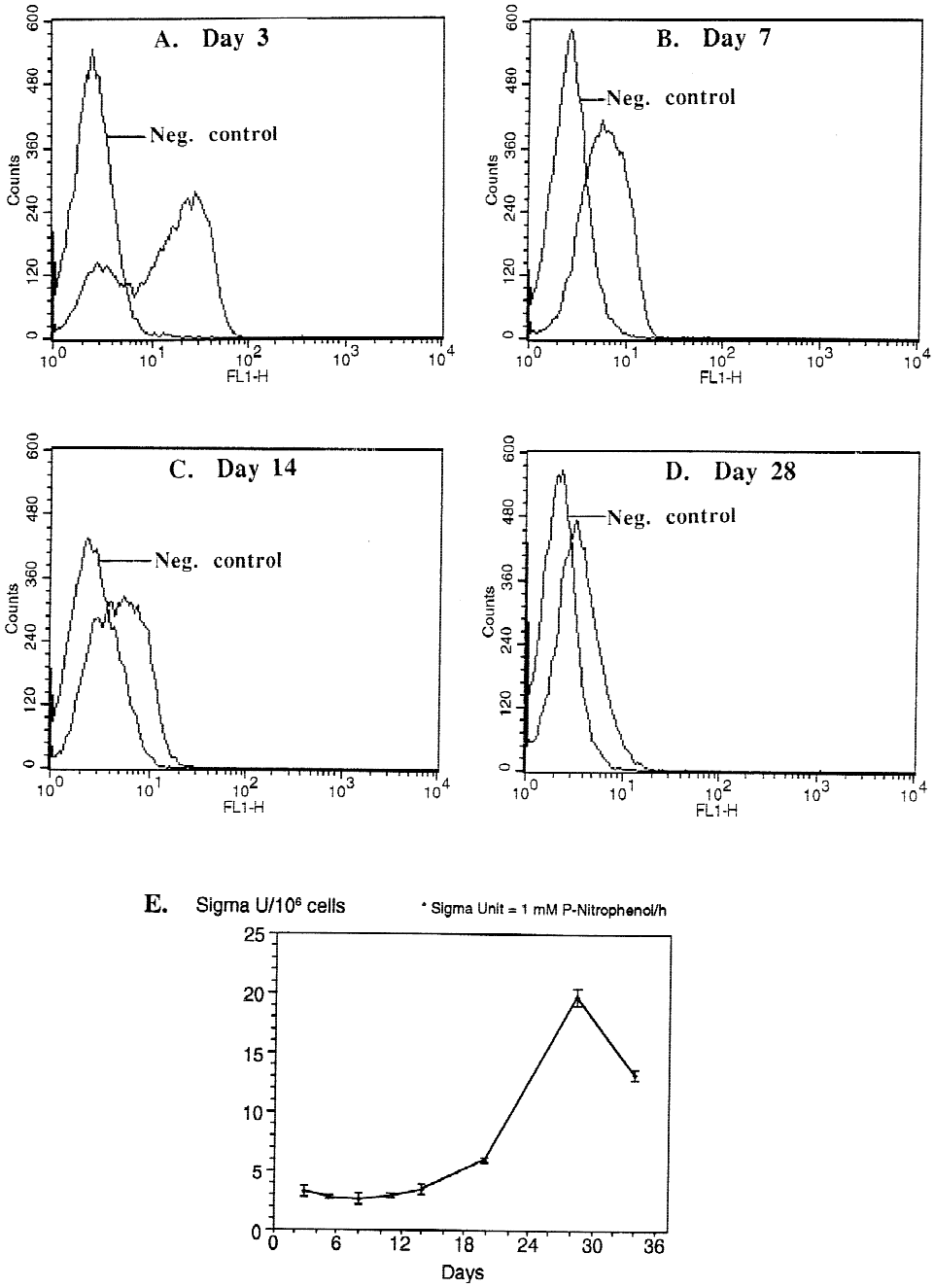

FIG. 3. Thy-1 expression by differentiating murine calvarial osteoblasts. Calvarial cells harvested from 3- to 5-day-old C57BL/6 mice were seeded into 100-mm dishes (1000–2000 cells/cm^2). Once confluence was reached (approximately day 7), the cultures were fed twice weekly with medium containing 2 mM β-glycerophosphate. The cells were collected from cultures on days 3, 7, 14, and 28 after seeding, and tested for Thy-1 expression by flow cytometry (A–D). Neg. control, the cells stained with secondary antibody only served as negative control. (E) ALP activity was determined at days 3, 5, 7, 14, 28, and 35. The ALP value was calculated using standards and expressed by Sigma units/10^6 cells. One Sigma unit is equal to 1 μM p-nitrophenol/h. Data are presented as the mean ± SD.

shown). These data support the idea that proliferation correlates with high Thy-1 expression.

To determine whether this pattern would also occur in other primary cell populations, the ABCs were examined next. The bone cells that grew out from the adult cortical bone chips and formed a subconfluent monolayer were designated passage zero (P_0). P_0 cells were separated from the chips, reseeded into tissue culture dishes and allowed to form a subconfluent monolayer designated as passage one (P_1). Passage two (P_2) was obtained by repeating the same

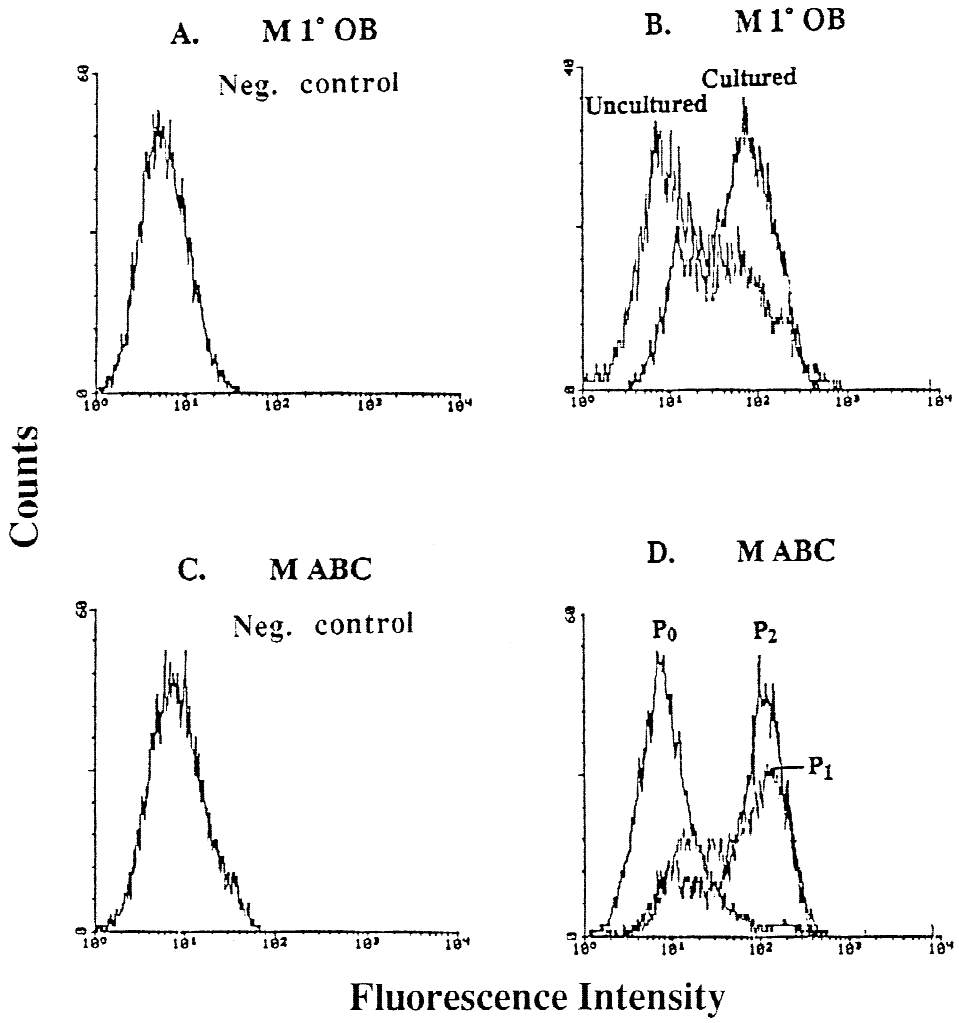

FIG. 4. Thy-1 expression by murine osteoblastic cells in subculture. Cells were stained with primary antibodies to Thy-1 antigen and a FITC-conjugated secondary antibody. (A) M 1°OB neg. control, primary neonatal murine osteoblastic cells (calvarial cells) were stained with secondary antibody only. (B) M 1°OB uncultured, the primary osteoblastic cells were collected from 3-day-old mice and analyzed for Thy-1 expression without culture; cultured, the cells were collected from 3-day-old mice and cultured until subconfluent (5 days) and then Thy-1 expression was determined. (C) M ABC neg. control, murine ABCs were stained with secondary antibody only. (D) M ABC, P_0 (passage zero), the ABCs were originally grown from the bone chips and cultured to form a confluent monolayer; P_1 (passage 1), P_0 ABCs were transferred once (2000 cells/cm^2) and grown in tissue culture plates to form a confluent monolayer; P_2 (passage two), P_1 cells were transferred once (2000 cells/cm^2) and grown to confluence.

procedure as P_1. In a pattern similar to that of primary calvarial osteoblasts, ABCs collected at P_0 were < 10% Thy-1$^+$, but expression increased to 40% in subconfluent P_1 and 60% in subconfluent P_2 (Figs. 4C and 4D). These data suggest that increasing Thy-1 expression may be a common feature of proliferating primary osteoblastic cells in culture.

Although unlikely, it was possible that the anti–Thy-1 antibody cross-reacted with an unknown epitope other than Thy-1 on osteoblastic cell surfaces. To address this possibility, two additional MAbs, JlJ and a FITC-conjugated rat anti-mouse Thy-1 antibody, were used to stain calvarial osteoblastic cells grown to subconfluence. Cells stained with either antibody were greater than 90% Thy-1$^+$ with a single discrete peak of brightly stained cells (data not shown). This pattern was not substantially different from that seen using G7.4.2 antibody. These data strongly suggest that the epitopes recognized by the antibodies were in fact on the Thy-1 molecule and that Thy-1 was expressed by cells in the osteoblastic lineage.

Effects of osteotropic agents and culture on Thy-1 expression

Previously, we have reported that the expression of another GPI-linked osteoblast differentiation antigen, Ly-6, could be modulated by exposure to specific osteotropic agents including BMP and 1,25$(OH)_2D_3$.[14] To determine

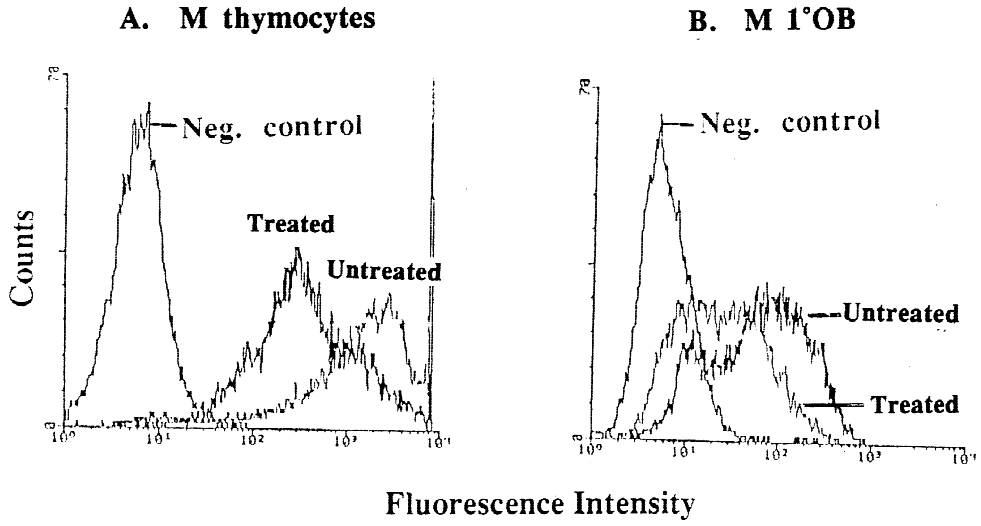

FIG. 5. Effect of PI-PLC treatment on Thy-1 expression by murine osteoblastic cells. Cells were treated with 1 U/ml PI-PLC for 90 minutes at 37°C, washed, and examined for Thy-1 expression using FACS analysis. Neg. Control, cells stained with secondary antibody only served as the negative control; untreated, no PI-PLC used; treated, cells treated with PI-PLC. (A) Murine thymocytes were used as a cellular positive control for PI-PLC treatment; (B) M 1°OB, primary neonatal murine osteoblastic cells.

whether similar treatment could also modify Thy-1 expression, subconfluent cultures of the MLB clones, calvarial osteoblasts, and ABCs were treated with BMP-2 (100 ng/ml), $1,25(OH)_2D_3$ (10 ng/ml), retinoic acid (10^{-5} M), or hydrocortisone (10^{-7} M) for 48 h and analyzed for Thy-1 expression by flow cytometry. Untreated cells served as a control. In none of the cells treated by any of the reagents under any circumstances was a change in Thy-1 expression observed (data not shown).

Sensitivity of osteoblast-expressed Thy-1 to treatment with PI-PLC

In thymocytes and T cells, Thy-1 is anchored to the plasma membrane by a GPI linkage. To determine whether Thy-1 was similarly anchored to the osteoblast cell surface, $1–5 \times 10^6$ neonatal murine calvarial cells or murine thymocytes (control) were treated in 1 ml with 1 U of PI-PLC for 90 minutes at 37°C. The cells were washed, stained with anti–Thy-1 antibody (G7.4.2), and analyzed by flow cytometry. Data in Fig. 5 show that both the calvarial cells and thymocytes treated with PI-PLC were reduced ~64% and 80%, respectively, in fluorescence intensity for Thy-1. This suggests that Thy-1 antigen is GPI linked to the osteoblasts' surface as it is to thymocytes'.

Thy-1 antigen expression by primary rat and human osteoblasts

It was possible that Thy-1 antigen expression was restricted to cells in the osteoblast lineage of the mouse. To determine if this was the case, primary fetal rat calvarial cells and primary human osteoblasts isolated from trabecular bone were assessed for Thy-1 expression. Primary fetal rat calvarial osteoblasts were isolated, grown to subconfluence, and tested for Thy-1 expression. The data in Fig. 6A show that 80% of these cells are Thy-1^+. These cells were similar in overall heterogeneity to primary mouse calvarial cells for the amount of antigen present on the cells as indicated by the broad range of fluorescent intensity, although there was a distinct shift to the right due to the numbers of brightly stained cells, indicative of increased antigen expression. Human osteoblasts were collected from subconfluent monolayers of out-growths from trabecular bone. Data shown in Fig. 6B demonstrate that essentially all (over 98%) of the human osteoblastic cells had Thy-1 on their surface with many of the cells expressing high levels of the antigen.

Thy-1 mRNA expression

To examine whether Thy-1 mRNA was detectable from the cells, total mRNA was isolated from MLB clones, subconfluent calvarial osteoblasts, and ABCs (P_0). The presence of mRNA encoding Thy-1 was determined by Northern blot analysis using thymocytes and spleen T cells as positive controls for Thy-1 mRNA. Data in Fig. 7 show the characteristic 1.8 kb band of Thy-1 mRNA in both thymocytes and spleen T cells. Thy-1 mRNA was not detected in MLB clone 14 (Fig. 7, lane 5) or clone 17 (lane 6) or in ABCs (lane 3). However, as in the positive controls, a band of mRNA of the appropriate molecular weight was detected in the calvarial osteoblast lane. Thy-1 mRNA could only be detected in calvarial osteoblasts, which correlates with the constitutive Thy-1 protein expression on the surface of these cells.

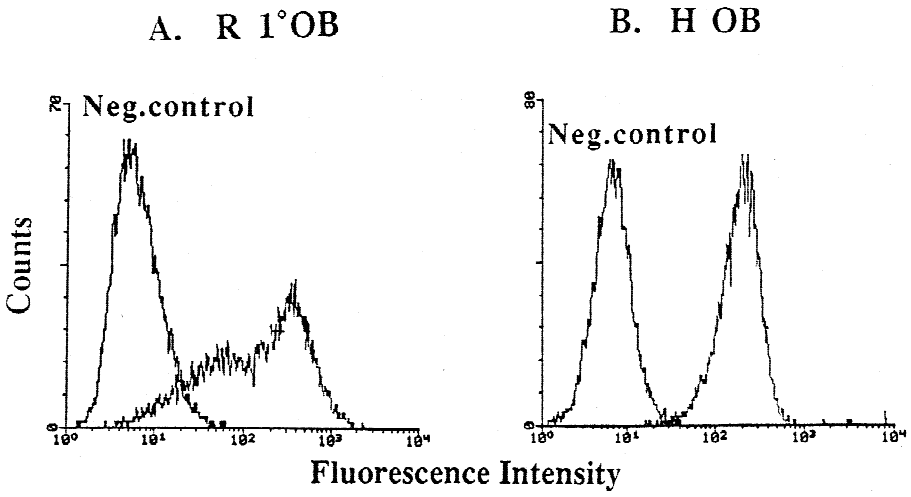

FIG. 6. FACS analysis of Thy-1 antigen expression by primary rat and human osteoblasts. Cells were grown to subconfluence, recovered by trypsin treatment, and stained with primary antibodies to Thy-1 antigen and a FITC-conjugated secondary antibody. Negative controls consisted of cells stained with secondary antibody only. (A) R 1°OB, primary raOBs harvested from calvariae of 22-day rat fetuses; (B) H OB, human trabecular bone osteoblastic cells.

DISCUSSION

In this report, we demonstrate for the first time expression by bone cells of the Thy-1 differentiation antigen. Thy-1 expression appears to change with the state of differentiation of cells in the osteoblast lineage. Constitutive expression is not seen on osteoblast progenitors, but can be detected on more mature calvarial osteoblast-like cells. However, cells from cortical bone, which have a more mature phenotype than calvarial osteoblastic cells, fail to express Thy-1. The highest level of constitutive expression was observed on proliferating cells and decreased once the cells progressed through the matrix maturation and mineralization stages. Thy-1$^+$ cells were observed on the endosteal surface of murine calvariae. Thy-1 expression was not species restricted and could be observed on primary mouse, rat, and human osteoblast-like cells. Steady-state mRNA correlated with cell surface expression in all cases.

The antigenic composition of the cell surface of osteoblasts, at any stage of differentiation, remains poorly characterized. Thy-1 is a differentiation antigen for cells in the T lymphocyte lineage. Expression is low on hematopoietic stem cells and high on both immature thymocytes and mature peripheral T lymphocytes in the mouse. Because we knew Thy-1 was expressed on relatively mature calvarial osteoblastic cells, we hypothesized that differences similar to those seen with T lymphocytes might be observed on cells in the osteoblast lineage. Stable clonal cell lines derived from 13 day postcoitus mouse limbs and marrow stromal fibroblasts, which have the capacity to differentiate into the bone lineage did not express detectable Thy-1. Bone marrow stromal cell lines (W20), which have the capacity to differentiate into osteoblasts under the influence of BMP also failed to express Thy-1. This is not, however, the case for all bone marrow stromal cell lines. Essentially all (93%) of the BMS-2 cells, which can differentiate into adipocytes

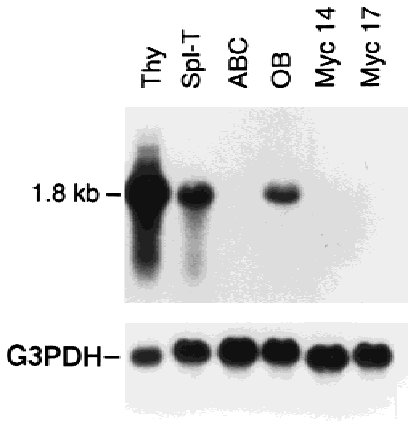

FIG. 7. Northern blot analysis of Thy-1 mRNA expression by murine osteoblast lineage cells. Total RNA was separated by electrophoresis, transferred to nylon membrane, and probed with a radiolabeled Thy-1 cDNA. The blot was stripped and reprobed with a radiolabeled cDNA for the housekeeping gene G3PDH (Clontech Laboratories, Palo Alto, CA, U.S.A.), shown in the lower panel, demonstrating equal RNA loading. Thy (lane 1), thymocytes (positive control); Spl-T (lane 2), spleen T lymphocytes (positive control); ABCs (lane 3), ABCs; OBs (lane 4), primary neonatal osteoblastic cells (calvarial cells); Myc 14 (lane 5), murine limb bud clone 14; Myc 17 (lane 6), murine limb bud clone 17.

and express osteoblast characteristics, expressed low levels of the antigen.[33] Freshly isolated calvarial cells (populations 3–5), which are enriched in osteoblast precursors and osteoblast-like cells and are more mature than the progenitor cells, were 45% positive for Thy-1 as determined by FACS analysis. The amount of antigen per cell was distributed over a wide range, encompassing no staining (negative

cells) to brightly stained cells (high levels of antigen). These data are in good agreement with the in vivo data showing Thy-1 expression by cells in close apposition to the bone surface and are morphologically identical to osteoblasts and bone lining cells, which are also in the osteoblast lineage.[47] It is difficult to know precisely why the lining cells are stained less brightly than the osteoblasts. One likely explanation is that the lining cells are less active and therefore express less antigen.

A much different pattern was observed on cells derived as outgrowths from adult cortical long bones. These cells expressed little (< 10%) Thy-1 antigen even after attaining subconfluence, which required at least 10 days in culture. Taken together, these data suggest that Thy-1 is not found on either early osteoprogenitors or on progenitor cells not committed to the osteoblast lineage. This is supported by the observation that osteoprogenitors isolated from murine bone marrow cells by flow cytometry expressed neither myeloid nor lymphoid markers, including Thy-1.[3] However, once these cells become committed to the osteoblast lineage, Thy-1 antigen may be expressed. The reason for the loss of antigen expression in the ABCs, which have characteristics of the most mature population, is not clear. One explanation is that expression is truly maturation dependent. This is supported by the data showing Thy-1 expression decreases with differentiation on hematopoietic stem cells and pre-B cells.[48] Alternatively, the fact that the cells are encased in bone and, by implication, are in a more quiescent state may account for the difference.

The changes observed in Thy-1 expression by the cultured calvarial cells are consistent with the idea that Thy-1 expression is linked to differentiation. Calvarial osteoblastic cells placed in culture mature through a series of stages characterized initially by increased proliferation with little matrix deposition, followed by decreased proliferation with increased matrix deposition, and finally mineralization.[37] Our data showed that Thy-1 expression decreased as the cells progressed from a less mature, low ALP, proliferating cell to a more mature cell high in ALP, and finally to a cell involved in mineralization. It appears that the critical transition is from proliferating to nonproliferating confluent cells. Thy-1 expression by both the proliferative and confluent calvarial cells was not affected by hydroxyurea treatment, suggesting that the expression of Thy-1 antigen was cell cycle independent. These data support our idea that Thy-1 expression is associated with osteoblast maturation. This reduction of Thy-1 antigen on the most mature cultured calvarial cells is consistent with the data showing that ABCs express low levels of detectable Thy-1 antigen.

We have made numerous attempts to modulate Thy-1 expression with a variety of osteotropic agents using multiple protocols with no success. This is similar to the lack of regulation of Thy-1 seen in lymphocytes and in contrast to the documented changes in Ly-6 expression induced with agents such as interferon, transforming growth factor-β, and $1,25(OH)_2D_3$.[14] A number of explanations may account for these data. As an example, although some cells may respond to BMP by an increase in ALP, this signal alone may not be sufficient to stimulate Thy-1 expression; additional or alternative signals, which may be provided in vivo, may be required to change Thy-1 expression. A cascade of signals in a specific order may be required to induce expression.

The fact that ABCs expressed significantly higher levels of Thy-1 antigen after being cultured for several generations, as compared with cells initially cultured to subconfluence, suggests three explanations. First, osteoblastic cells from adult mice might be induced to "de-differentiate," taking on a phenotype of a less mature, proliferating cell, in this case a phenotype similar to neonatal mouse calvarial osteoblasts. The de-differentiation alternative does not fit well with the known data. Terminally differentiated cells, like osteocytes, rarely re-enter the cell cycle spontaneously, and must be forced to do so by some external manipulation such as infection with DNA tumor virus oncogenes. The data showing that ABCs express low Thy-1 and proliferate poorly in vitro also fails to support the de-differentiation explanation.[49] Second, ABCs may contain a small population of resting precursors which are triggered to proliferate and differentiate into Thy-1^+ cells in culture. This explanation agrees with the data that demonstrated that increased proliferation and Thy-1 expression are coincident for differentiating calvarial cells. Moreover, our preliminary data suggest that a small population of highly proliferative cells which express high levels of Thy-1 antigen may be isolated from ABCs.[50] A third alternative is that it is possible that the change in Thy-1 expression is an artifact of in vitro culture. However, this seems unlikely because culturing of these cells also results in an increase in Ly-6 expression and, more importantly, a decrease in the expression of certain class I antigens which map outside of the mouse major histocompatibility complex (data not shown). We speculate that at least part of the signal(s) which allows these cells to change their phenotype, regardless of the three explanations stated above, is their release from their bony sarcophagi and exposure to the external milieu. This speculation is supported by the data showing numerous changes in bone cell activity in vivo and in vitro following fracture, which also exposes these cells to the external environment.[51]

Thy-1 is similar to Ly-6 in that they are both anchored to the cell surface through a GPI linkage. The fact that Thy-1 can be cleaved from the osteoblastic surface by treatment with PI-PLC places Thy-1 in the same class of GPI-linked antigens as ALP, the receptor for urokinase-type plasminogen activator (uPAR) and an antigen which maps to the right of the D region of the murine major histocompatibility complex, possibly in the Qa region (M. Horowitz, unpublished data). uPAR is similar or identical to the osteoblast growth factor derived from prostate tumors which is responsible for skeletal metastases.[52] uPAR and Ly-6 are related based on sequence analysis, suggesting that they may have arisen from a common ancestral gene.[53] Therefore, it appears that Thy-1 is a member of a family of GPI-linked antigens which are expressed on the cell surface of osteoblasts, at different stages of maturation, and which may regulate cell activation.

It is generally accepted that as cells in the osteoblast lineage mature they are found in closer proximity to bone surfaces.[54] This agrees with the in vivo data demonstrating

that cells expressing Thy-1 were found predominately on the endosteal surface and supports the observation that Thy-1 is not present on early osteoblast progenitors. The fact that late osteoblast precursors and osteoblast-like cells (e.g., mouse calvarial, fetal rat, and human trabecular bone cells) express high levels of Thy-1, but mature cells in bone do not (ABCs), suggests that these bone surface associated cells may be separated from both their surrounding bone marrow as well as bone matrix cells by expression of Thy-1 and, furthermore, that this marker may be used to follow osteoblast development in vivo. As in the case for T cells and other cell types, use of cell surface antigens alone or in combination with other markers provides a potentially powerful method to isolate and characterize these cells in regard to their state of differentiation, pathway of development, and functional activity.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Pamela Robey and Roland Baron for their support of this work. We thank Ms. Andrea Fields and Mr. Dylan Distasio for their careful review of the manuscript. Special thanks to Genetics Institute for providing BMP. This work was supported by National Institutes of Health Grants AR-40073 and AR-40507 and the Department of Orthopaedics and Rehabilitation, Yale University School of Medicine.

REFERENCES

1. Bab I, Passu-Even L, Gazit D, Sekeles E, Ashton B, Peylan-Ramu N, Ziv I, Ulmansky M 1988 Osteogenesis in in vivo diffusion chamber cultures of human marrow cells. Bone Miner **4:**373–386.
2. Beresford J 1989 Osteogenic stem cells and the stromal system of bone and marrow. Clin Orthop **240:**270–280.
3. Van Vlasselaer P, Falla N, Snoeck H, Mathieu E 1994 Characterization and purification of osteogenic cells from murine bone marrow by two-color cell sorting using anti–Sca-1 monoclonal antibody and wheat germ agglutinin. Blood **84:**753–763.
4. Bruder SP, Caplan AI 1990 Terminal differentiation of osteogenic cells in the embryonic chick tibia is revealed by a monoclonal antibody against osteocytes. Bone **11:**189–198.
5. Aubin JE, Turksen K 1996 Monoclonal antibodies as tools for studying the osteoblastic lineage. Microsc Res Tech **33:**128–140.
6. Bruland ØS, Fodstad Ø, Stenwig AE, Pihl A 1988 Expression and characteristics of a novel human osteosarcoma-associated cell surface antigen. Cancer Res **48:**5302–5309.
7. Bruder SP, Caplan AI 1989 First bone formation and the dissection of an osteogenic lineage in the embryonic chick tibia is revealed by monoclonal antibodies against osteoblasts. Bone **10:**359–375.
8. Noda M, Yoon K, Rodan GA, Koppel DE 1987 High lateral mobility of endogenous and transfected alkaline phosphatase: A phosphatidylinositol-anchored membrane protein. J Cell Biol **105:**1671–1677.
9. Turksen K, Aubin JE 1991 Positive and negative immunoselection for enrichment of two classes of osteoprogenitor cells. J Cell Biol **114:**373–384.
10. Nijweide PJ, Mulder RJP 1986 Identification of osteocytes in osteoblast-like cell cultures using a monoclonal antibody specifically directed against osteocytes. Histochemistry **84:**342–347.
11. Hughes DE, Salter DM, Simpson R 1994 CD44 expression in human bone: A novel marker of osteocytic differentiation. J Bone Miner Res **9:**39–44.
12. Yamaguchi A, Kahn AJ 1993 Monoclonal antibodies that recognize antigens in human osteosarcoma cells and normal fetal osteoblasts. Bone Miner **22:**165–175.
13. Van Der Plas A, Nijweide PJ 1992 Isolation and purification of osteocytes. J Bone Miner Res **7:**389–396.
14. Horowitz MC, Fields A, DeMeo D, Qian H-Y, Bothwell ALM, Trepman E 1994 Expression and regulation of Ly-6 differentiation antigens by murine osteoblasts. Endocrinology **135:**1032–1043.
15. Palfree RG, Hämmerling U 1986 Biochemical characterization of the murine activated lymphocyte alloantigen Ly-6E.1 controlled by the Ly-6 locus. J Immunol **136:**594–600.
16. Reiser H, Yeh ETH, Gramm CF, Benacerraf B, Rock KL 1986 Gene encoding T-cell–activating protein TAP maps to the Ly-6 locus. Proc Natl Acad Sci USA **83:**2954–2958.
17. Shevach EM, Korty PE 1989 Ly-6: A multigene family in search of a function. Immunol Today **10:**195–200.
18. Van de Rijn M, Heimfeld S, Spangrude GJ, Weissman IL 1989 Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. Proc Natl Acad Sci USA **86:**4634–4638.
19. Bruder SP, Horowitz MC, Haynesworth SE 1996 Monoclonal antibodies selective for human osteogenic cell surface antigens. Bone **21:**225–235.
20. Bruder SP, Ricalton NS, Boynton RE, Connolly TJ, Barry FP 1997 Immunopurification, peptide sequence analysis, and molecular cloning of a human osteoprogenitor cell surface antigen. Transactions of the 43rd Annual Meeting of the Orthopaedic Research Society, San Francisco CA, U.S.A., pp. 13 (abstract).
21. McKenzie JL, Fabre JW 1981 Human Thy-1: Unusual localization and possible functional significance in lymphoid tissue. J Immunol **126:**843–850.
22. Low MG, Kincake PW 1985 Phosphatidylinositol is the membrane- anchoring domain of the Thy-1 glycoprotein. Nature **318:**62–64.
23. Blankenhorn EP, Douglas TC 1972 Location of the gene for Theta antigen in the mouse. J Hered **63:**259–263.
24. Williams AF, Gagnon J 1982 Neuronal cell Thy-1 glycoprotein: Homology with immunoglobulin. Science **216:**696–703.
25. Reif AE, Allen JMV 1964 The AKR thymic antigen and its distribution in leukemias and nervous tissue. J Exp Med **120:**413–433.
26. Spangrude GJ, Heimfeld S, Weissman IL 1988 Purification and characterization of mouse hematopoietic stem cells. Science **241:**58–60.
27. Uchida N, Weissman IL 1992 Searching for hematopoietic stem cells: Evidence that Thy-1.1^{lo} Lin^{-} Sca-1^{+} cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. J Exp Med **175:**175–184.
28. Gunter KC, Malek TR, Shevach EM 1984 T cell-activating properties of an anti–Thy-1 monoclonal antibody. J Exp Med **159:**716–730.
29. Rosen V, Nove J, Song JJ, Thies RS, Cox K, Wozney JM 1994 Responsiveness of clonal limb bud cell lines to bone morphogenetic protein 2 reveals a sequential relationship between cartilage and bone cell phenotypes. J Bone Miner Res **9:**1759–1768.
30. Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM 1992 Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20–17 stromal cells. Endocrinology **130:**1318–1324.
31. Pietrangeli CE, Hayashi S-I, Kincade PW 1988 Stromal cell lines which support lymphocyte growth: Characterization, sensitivity to radiation and responsiveness to growth factors. Eur J Immunol **18:**863–872.
32. Gimble JM, Hudson J, Henthorn J, Hua X, Burstein SA 1991 Regulation of interleukin 6 expression in murine bone marrow stromal cells. Exp Hematol **19:**1055–1060.
33. Dorheim MA, Sullivan M, Dandapani V, Wu X, Hudson J,

Segarini PR, Rosen DM, Aulthouse AL, Gimble JM 1993 Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. J Cell Physiol **154:**317–328.
34. Kuznetsov S, Robey PG 1996 Species differences in growth requirements for bone marrow stromal fibroblast colony formation in vitro. Calcif Tissue Int **59:**265–270.
35. Centrella M, McCarthy TL, Canalis E 1987 Transforming growth factor beta is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat calvariae. J Biol Chem **262:**2869–2874.
36. Centrella M, McCarthy TL, Canalis E 1989 Platelet-derived growth factor enhances deoxyribonucleic acid and collagen synthesis in osteoblast-enriched cultures from fetal rat parietal bone. Endocrinology **125:**13–19.
37. Stein GS, Lian JB 1993 Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev **14:**424–442.
38. Robey PG, Termine JD 1985 Human bone cells in vitro. Calcif Tissue Int **37:**453–460.
39. McCarthy TL, Centrella M, Canalis E 1988 Further biochemical and molecular characterization of primary rat parietal bone cell cultures. J Bone Miner Res **3:**401–408.
40. Levy JB, Schindler C, Raz R, Levy DE, Baron R, Horowitz MC 1996 Activation of the JAK-STAT signal transduction pathway by oncostatin-M in cultured human and mouse osteoblastic cells. Endocrinology **137:**1159–1165.
41. Gunter KC, Malek TR, Shevach EM 1984 T cell activating properties of an anti–Thy-1 monoclonal antibody: Possible analogy to OKT3/Leu-4. J Exp Med **159:**716–730.
42. Bruce J, Symington FW, McKearn TJ, Sprent J 1981 A monoclonal antibody discriminating between subsets of T and B cells. J Immunol **127:**2496–2501.
43. Su B, Wanec GL, Flavell RA, Bothwell ALM 1991 The glycosylphosphatidylinositol anchor is critical for Ly-6A/E-mediated T cell activation. J Cell Biol **112:**377–384.
44. Horne WC, Neff L, Chatterjee D, Lomri A, Levy JB, Baron R 1992 Osteoclasts express high levels of pp. $60^{c\text{–}src}$ in association with intracellular membranes. J Cell Biol **119:**1003–1013.
45. Church GM, Gilbert W 1984 Genomic sequencing. Proc Natl Acad Sci USA **81:**1991–1995.
46. Wysocki LJ, Sato VL 1978 'Panning' for lymphocytes: A method for cell selection. Proc Natl Acad Sci USA **75:**2844–2848.
47. Baron RE 1996 Anatomy and ultrastructure of bone. In: Favus MJ (ed.) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 3rd Ed. Lippincott-Raven, Philadelphia, PA, U.S.A., pp. 3.
48. Shevach EM 1989 Accessory Molecules. In: Paul WE (ed.) Fundamental Immunology, 2nd Ed. Raven Press, New York, NY, U.S.A., pp. 431.
49. Chen X-D, Horowitz MC 1996 Identification of osteoprogenitors in murine long bone. J Bone Miner Res **11:**T352 (abstract).
50. Chen X-D, Horowitz MC 1997 Isolation of osteoprogenitors from murine long bone by three-color FACS. J Bone Miner Res **12**(suppl):S405 (abstract).
51. Bolander ME 1992 Regulation of fracture repair by growth factors. Proc Soc Exp Biol Med **200:**165–170.
52. Koutsilieris M, Rabbani SA, Bennett HPJ, Goltzman D 1987 Characteristics of prostate-derived growth factors for cells of the osteoblast phenotype. J Clin Invest **80:**941–946.
53. Palfree GE 1991 The urokinase-type plasminogen activator receptor is a member of the Ly-6 superfamily. Immunol Today **12:**170–171.
54. Puzas JE 1996 Osteoblast cell biology. In: Favus MJ (ed.) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 3rd Ed. Lippincott-Raven, Philadelphia, PA, U.S.A., pp. 11.

Address reprint requests to:
Dr. Mark Horowitz
Department of Orthopaedics and Rehabilitation
Yale University School of Medicine
P.O. Box 208071
New Haven, CT 06520-8071 U.S.A.

Received in original form April 8, 1998; in revised form September 25, 1998; accepted October 21, 1998.